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Mass spectrometry based investigation of Alzheimer's disease and its biomarkers

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Stockholm 2013

Published by Karolinska Institutet.

Printed by Larserics Digital Print AB

ISBN 978-91-7549-271-1

TO MY PARENTS

致我的父母

“A TIDY LABORATORY MEANS A LAZY CHEMIST.”

Jöns Jakob Berzelius

ABSTRACT

The overall aim of this work was to find biomarkers for Alzheimer's disease (AD) in such an easily accessible body fluid as blood and investigate the implications of these biomarkers for the AD mechanism. The central and most studied histopathological features in AD brain are the amyloid plaques. Even at the preclinical stage of AD, amyloid beta (Aβ) peptides already start to form plagues. It still remains unclear whether Aβ oligomers and fibrils initiate the molecular cascade that ultimately leads to AD. So far, all Aβ-centered therapy approaches have had little success. Therefore, a fresh, unorthodox look at the AD initiation and biomarkers of the disease development is required.

Since the primary risk factor for AD is age, biomarkers of protein aging could hypothetically be linked to AD. One of the main protein aging pathways is isoaspartate (isoAsp) accumulation; increased isoAsp levels are found in AD amyloid plaques. Thus we developed a mass spectrometry-based platform and workflow for isoAsp detection and quantification (Paper I), and applied them for the analysis of blood plasma samples from AD patients and healthy controls. We found increased levels of isoAsp in all stages of dementia (Paper II), which supported the hypothetical mechanism in which isoAsp accumulation triggers a sequence of events resulting in AD. To further investigate the impact of isoAsp accumulation in the central nervous system, we analyzed the brains of the transgenic mouse model lacking isoAsp repair via methylation (Paper III), and found that, along with the increased isoAsp levels, glutamate pathway is seriously distorted. IsoAsp can be cleared from the organism, besides repair, by means of proteases. When analyzing protein abundances in blood, we found links between these protein functions and AD development (Paper IV). The changes in the protein abundances were small, but nevertheless predictive of the AD progression. How early can one detect the imminent AD development by protein biomarkers? To address this question in the best possible scenario (a well-controlled system, brain biopsy), we analyzed the brain proteome of young transgenic mice and found statistically significant changes occurring long before the onset of AD-like symptoms (Paper V). Therefore, protein abundances, together with isoAsp levels, are promising biomarker candidates for AD diagnostics and prognostics, and mass spectrometry is an adequate tool for analyzing them.

LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. **Hongqian Yang**, Eva Y. M. Fung, Alexander R. Zubarev and Roman A. Zubarev. *Toward proteome-scale identification and quantification of isoaspartyl residues in biological samples,* J. Proteome Res., 2009, 8(10): 4615-21.

II. **Hongqian Yang**, Yaroslav Lyutvinskiy, Hilkka Soininen and Roman A. Zubarev. *Alzheimer's disease and mild cognitive impairment are associated with elevated levels of isoaspartyl residues in blood plasma prot*eins, J. Alzheimers Dis., 2011, 27(1): 113-8.

III. **Hongqian Yang**, Jonathan D. Lowenson, Steven Clarke, and Roman A. Zubarev. *Brain proteomics supports the role of glutamate metabolism and suggests other metabolic alterations in protein L-isoaspartyl methyltransferase (PIMT)-knockout mice*, J. Proteome Res., accepted.

IV. **Hongqian Yang**, Yaroslav Lyutvinskiy, Sanna-Kaisa Herukka, Hilkka Soininen, Dorothea Rutishauser and Roman A. Zubarev. *Prognostic polypeptide blood plasma biomarkers of Alzheimer's disease progression*, manuscript.

V. **Hongqian Yang**, Jessica L. Wittnam, Roman A. Zubarev and Thomas A. Bayer. *Shotgun brain proteomics reveals early molecular signature in presymptomatic mouse model of Alzheimer's disease*, J. Alzheimers Dis., in press.

PUBLICATIONS NOT INCLUDED IN THIS THESIS

I. **Hongqian Yang** and Roman A. Zubarev. *Mass spectrometric analysis of asparagine deamidation and aspartate isomerization in polypeptides*, Electrophoresis, 2010, 31(11): 1764-72.

II. **Hongqian Yang** and Roman A. Zubarev. *Recognition of amino acid chirality in polypeptide ions by MS/MS, Chiral Recognition in the Gas Phase*, Anne Z, ed, Taylor and Francis Group, ISBN 978-1-4200822-7-2, 2010.

III. Roman A. Zubarev and **Hongqian Yang**. *Multiple soft ionization of gas-phase proteins and swift backbone dissociation in collisions with ≤ 99 eV electrons*, Angew. Chem. Int. Ed., 2010, 49(8): 1439-41.

IV. Mikhail V. Gorshkov, David M. Good, Yaroslav Lyutvinskiy, **Hongqian Yang** and Roman A. Zubarev. *Calibration function for the Orbitrap FTMS accounting for the space charge effect*, J. Am. Soc. Mass. Spectrom., 2010, 21(11): 1846-51.

V. Roman A. Zubarev, Konstantin A. Artemenko, Alexander R. Zubarev, Corina Mayrhofer, **Hongqian Yang** and Eva Y. M. Fung. *Early life relict feature in peptide mass distribution*, Cent. Eur. J. Biol., 2010, 5(2): 190-6.

VI. Atim A. Enyenihi, **Hongqian Yang**, A. Jimmy Ytterberg, Yaroslav Lyutvinskiy, and Roman A. Zubarev. *Heme binding in gas-phase holo-myoglobin cations: distal becomes proximal?* J. Am. Soc. Mass. Spectrom., 2011, 22(10): 1763-70.

VII. Philippe Grappin, Boris Collet, **Hongqian Yang**, Denis Jallet, Laurent Ogé, and Roman A. Zubarev. *New proteomic developments to analyze protein isomerization and their biological significance in plants*, J. Proteomics, 2011, 74(8): 1475-82.

VIII. **Hongqian Yang**, David M. Good, David van der Spoel and Roman A. Zubarev. *Carbonyl charge solvation patterns may relate to fragmentation classes in collision-activated dissociation*, J. Am. Soc. Mass. Spectrom., 2012, 23(8): 1319-25.

IX. Yordanka Masforrol, Jeovanis Gil, Luis Javier Gonzalez, Yasset Perez-Riverol, Jorge Fernandez-de-Cossio, Aniel Sanchez, Lazaro Hiram Betancourt, Hilda Elisa Garay, Ania Cabrales, Fernando Albericio, **Hongqian Yang**, Roman A. Zubarev, Vladimir Besada, and Osvaldo Reyes Acosta. *Introducing an Asp-Pro linker in the synthesis of random one-bead-one-compound hexapeptide libraries compatible with ESI-MS analysis*, ACS Comb. Sci., 2012, 14(3): 145-9.

X. Yaroslav Lyutvinskiy, **Hongqian Yang**, Dorothea Rutishauser, and Roman A. Zubarev. *In silico instrumental response correction improves precision of label-free proteomics and accuracy of proteomics-based predictive models*, Mol. Cell. Proteomics, 2013, 12(8): 2324-31.

XI. David M. Good, **Hongqian Yang**, and Roman A. Zubarev. *N-terminal peptide sequence repetition influences the kinetics of backbone fragmentation: a manifestation of the Jahn-Teller Effect?* J. Am. Soc. Mass. Spectrom., in press.

XII. Susanna L. Lundström, **Hongqian Yang**, Yaroslav Lyutvinskiy, Dorothea Rutishauser, Sanna-Kaisa Herukka, Hilkka Soininen and Roman A. Zubarev. *Blood plasma IgG Fc glycans are significantly altered in Alzheimer's disease and progressive mild cognitive impairment*, J. Alzheimers Dis., accepted.

CONTENTS

ABBREVIATIONS

A2M	α-2-macrogloblin
AD	Alzheimer's disease
AdoMet	S-adenosylmethionion
Asn	asparagine residue
Asp	aspartate residue
Aβ	amyloid beta
$A\beta_{pE3-42}$	$A\beta_{3-42}$ with pyroglutamate at the third position
CAD	collisional activated dissociation
CSF	cerebrospinal fluid
CV	coefficient of variability
ECD	electron capture dissociation
ETD	electron transfer dissociation
FASP	filter-aided sample preparation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HCD	higher-energy C-trap dissociation
HPLC	high performance liquid chromatography
$InsP_3$	inositol-1,4,5-trisphosphate
isoAsp	isoaspartic acid residue
$InsP_3KA$	inositol-trisphosphate 3-kinase A
KO	knockout
LC	liquid chromatography
LTQ	linear trap quadrupole
m/z	mass over charge ratio
MCI	mild cognitive impairment
MS	mass spectrometry
MS/MS	tandem mass spectrometry
mTOR	mammalian target of rapamycin
Necl 1	nectin-like molecule 1
NFTs	neurofibrillary tangles
OPLS-DA	orthogonal partial least squares discriminant analysis
PCA	principal component analysis
PIMT	protein L-isoaspartyl methyltransferase
P-MCI	progressive mild cognitive impairment
S-MCI	stable mild cognitive impairment
WT	wild type

1 INTRODUCTION

1.1 ALZHEIMER'S DISEASE

1.1.1 Background

Alzheimer's disease (AD) is a severe neurodegenerative disease and the main cause of dementia. There are more than 35 million dementia patients worldwide, and the number is estimated to reach 115 million by the year 2050.[1] AD gradually affects the cognitive and everyday executive abilities of the patients, with advanced ones requiring help with the basic activities of daily living and ultimately dying of the disease.[2] It is estimated that the initiation of AD begins 10-15 years before the clinical onset, and the clinical progression rates differ among AD patients.[3] In the clinics, the diagnosis of AD relies on medical history, physical and neurological examination, as well as structural and functional brain imaging techniques. According to Alzheimer's Association, the AD experts can diagnose the disease with more than 90% accuracy. Currently, the definite diagnosis can only be achieved in postmortem brain examination, while the new AD definition proposed by the International Working Group, which allows accurate *in vivo* diagnosis of AD, remains to be commonly adopted by the clinical community.[4] Currently there is no treatment to prevent or cure AD, which might be partially due to the lack of early and accurate clinical diagnosis.[3] To address these issues, in the year 2011 the National Institute on Aging defined three stages of AD: dementia due to AD; mild cognitive impairment (MCI) due to AD; and preclinical AD, and recommended implementing biomarkers as a complementary diagnosis tool.[5] MCI is the stage where patients experience more memorial impairment compared with healthy controls while less cognitive impairment compared with mild AD patients.[6] On average, the risk of these MCI patients progressing into AD increases 10-12% every year, which is faster compared with healthy subjects. However, not every MCI patient will develop AD.[6] It remains challenging to predict the clinical outcome for the MCI patients.

1.1.2 The amyloid cascade hypothesis

The molecular pathognomonic hallmarks of AD brain include the deposition of extracellular amyloid plaques composed of amyloid beta (Aβ) peptides and the formation of intracellular neurofibrillary tangles (NFTs) composed of hyper-phosphorylated tau proteins. Although the molecular linkage between Aβ accumulation

and tau pathology remains unclear, it is generally accepted that tau pathology is downstream of Aβ accumulation in AD progression.[7, 8] Amyloid cascade hypothesis, which suggests that the imbalance between Aβ production and clearance causes the aggregation of Aβ peptides, and thus triggers the process of neurodegeneration, proposes Aβ accumulation as the primary event in AD pathogenesis.[8] There are many experimental observations that support this hypothesis, e.g. the fact that the genetic mutations that perturb Aβ metabolism can lead to early onset of AD, with the upstream Aβ oligomers being more neurotoxic than the plagues themselves.[8] But there are also observations that cannot be explained by the hypothesis, such as the poor correlation between the brain amyloid loads and the degree of patients' cognitive impairment. Moreover, many healthy subjects have substantial amyloid deposits in their brains.[9, 10] There are many forms of Aβ peptides found in AD brains, but it still remains unclear which isoform(s) of Aβ initiate(s) the formation of the neurotoxic soluble Aβ oligomers.[11] The N-terminal truncated form with pyroglutamate at the third position, $A\beta_{pE3\text{-}42}$, has more than 2-fold and up to 250-fold higher propensity to form oligomers than $A\beta_{40}$ and $A\beta_{42}$ under several different conditions *in vitro*.[12, 13] Thus it is possible that $A\beta_{pE3\text{-}42}$ forms oligomers earlier than other Aβ peptides *in vivo*.

To investigate the role of Aβ, many transgenic mouse models have been developed, however, none of them can fully cover the comprehensive neuropathology of human AD brain.[14] For AD amyloid studies, there are several mouse strains established, e.g. PDAPP,[15] Tg2576,[16] J20,[17] APP23,[18] TgCRND8,[19] and 5XFAD mice.[20] While all of these mouse models form amyloid plaques, none show NFTs in the brain.[14] These transgenic mice develop amyloid plaques at different time points with various amyloid forms (diffused or condensed), and many of them do not show significant neuron loss in the brain.[14] Therefore, the animal models should be chosen according to the specific aims of the research.

The TBA42 mouse model that exclusively expresses $A\beta_{pE3\text{-}42}$ in the brain exhibits neurodegeneration without any apparent plagues and tangles, which confirms the neurotoxicity of soluble $A\beta_{pE3\text{-}42}$.[21] Crossing this transgenic mouse model with a conventional AD model 5XFAD results in the increased amyloid loads in their offspring, which supports the "seeding" role of $A\beta_{pE3\text{-}42}$.[22] Thus the TBA42 mice may serve as a well-established model to investigate the early molecular events in AD.

1.1.3 Biomarkers of Alzheimer's disease

Although amyloid cascade is the central and most studied topic in AD research, there is little success in developing the Aβ-centered therapeutics.[8] As the preclinical stage of AD lasts more than one decade, the medical intervention timing is crucial. Currently AD cannot be confirmed by any single test, and the patients are mostly diagnosed at the late stage of the disease. Therefore AD biomarkers for early diagnosis and prognosis are of demand.

The ideal biomarkers should be reliable, non-invasive, and inexpensive. So far the most studied and validated biomarker candidates are the polypeptides in the cerebrospinal fluid (CSF). (The imaging biomarkers are also strong and promising candidates,[23] while they are out of the scope of this thesis.) $A\beta_{42}$, total tau, and phosphorylated tau are in use for discriminating AD versus healthy subjects with the sensitivity and specificity around 80-90%.[24] For predicting the clinical development of MCI patients, whether they will progress to AD (P-MCI) or remain stable as MCI (S-MCI), CSF biomarkers could achieve sensitivity and specificity around 80%,[25] although broader validation might be needed.[26, 27] However, obtaining CSF is invasive and CSF is not routinely analyzed for MCI patients in most of the countries.

Compared with CSF, obtaining blood is much less invasive and blood samples are routinely analyzed in the clinics. A number of studies has investigated blood samples in search for AD biomarkers in the past decade.[27] One of such studies identified a panel of 18 signaling plasma proteins that can differentiate AD versus healthy subjects with the sensitivity and specificity around 90%.[28] In this study, the combination of those 18 proteins could discriminate S-MCI and P-MCI with the sensitivity and specificity around 80%,[28] which is the best result achieved among this type of studies to date.

1.2 Isoaspartate

1.2.1 The origin of isoaspartate

Besides the amyloid hypothesis, there are other proposed disease mechanisms, including: the neurovascular hypothesis, the inflammatory mechanism, and the protein aging theory. These mechanisms are not necessarily mutually exclusive: for instance, protein aging hypothesis helps explaining what process initiates aggregation of polypeptides, including Aβ.[29] One of the major protein aging pathways is the accumulation of isoaspartic acid residues (isoAsp) in protein sequences.

IsoAsp is generated from either asparagine residue (Asn) deamidation or aspartic acid residue (Asp) isomerization through the succinimide intermediate. Succinimide is labile to hydrolysis which process results in a mixture of Asp and isoAsp with a typical ratio around 1:3 (Figure 1).[30] Both deamidation and isomerization are spontaneous reactions under physiological condition, and the reaction rate is influenced by protein primary sequence, protein structure, pH, temperature, and buffer ionic strength.[31] IsoAsp accumulates significantly with time in long-lived proteins, such as α-crystallin in eye lenses[32] and even albumin[33] in blood. However, the enzyme, protein L-isoaspartyl methyltransferase (PIMT) initiates the conversion of isoAsp into succinimide using S-adenosylmethionion (AdoMet) as the methyl donor, which will partially hydrolyze into Asp residues.

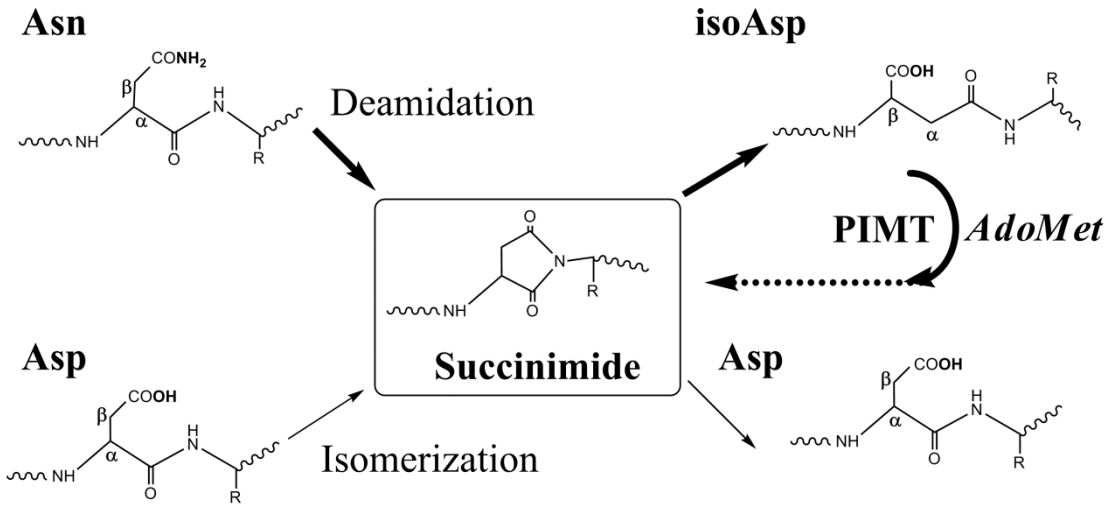

Figure 1. Asn deamidation and Asp isomerization reactions that generate isoAsp and Asp.

1.2.2 Biological significance of isoaspartate

Under physiological conditions, the isoAsp introduced site-specifically by deamidation and isomerization can have regulatory functions, such as promoting DNA damage-induced apoptosis[34] and suppressing p53 activity.[35] But mostly, the presence of isoAsp is damaging, as the rearrangement of CH_2 group from the Asn/Asp side chain to the backbone can disrupt protein structure and affect its function. IsoAsp accumulation that occurs in the natural process of aging usually has negative impact, e.g. isoAsp affects the stability of human lens[32] and can induce autoimmune response.[36] The introduction of isoAsp could also cause the polypeptide to become resistant to protease degradation.[37] Therefore, isoAsp formation and PIMT repair capability have essential roles in both cellular regulation and protein aging processes. In pharmaceutical

industry, minimizing deamidation and isomerization during drug production, formulation and storage is of great importance.[38]

To further explore the biological influence of isoAsp accumulation, transgenic mouse strain with the deletion of the gene encoding PIMT has been established.[39, 40] The deletion of PIMT gene causes isoAsp accumulation in all the tissues measured in the studies, especially in the brain.[39, 40] These PIMT-knockout (KO) mice have perturbed synaptosome glutamate uptake and die prematurely of epileptic seizures.[39] As the expression of PIMT is crucial for survival, the *in vivo* substrates of PIMT have been under intense studies.[41-44] Because of the strong physiological response, there are reasons to suspect that the PIMT knockout affects important brain pathways.[39, 40] So far, it has been reported that PIMT regulates p53 activity,[35] and the absence of PIMT results in activation of the insulin[45, 46] and MEK-ERK pathways.[47]

1.3 Isoaspartate and AD – the protein aging hypothesis

The protein aging hypothesis of AD reinforces the role of Aβ peptide by proposing that the cyclization of Asp in Aβ peptides initiates the aggregation that decades later results in the disease.[48] In a broader perspective, it is the deterioration (deamidation and isomerization) of polypeptides, including Aβ peptides as well as other amyloidogenic proteins that triggers protein aggregation, and thus leads to other downstream molecular cascades.[49] IsoAsp has been localized in Aβ peptides from amyloid plagues purified from AD brains at positions 1, 7 and 23.[50, 51] Also, higher amount of isoAsp has been identified in Aβ peptides as well as paired helical filaments of AD brain compared with healthy subjects.[52] However, the questions related to the identities of other proteins bearing isoAsp damages, and whether they are amyloidogenic, have been poorly investigated so far.[53]

2 AIMS OF STUDY

The aims of this Ph.D. thesis were to search for protein biomarkers of AD in blood and, if these are found, investigate the implications of these biomarkers for the AD mechanism. More specifically, the aims included:

1. to establish and optimize mass spectrometry-based platform and workflow for the study of isoAsp at the whole proteome level (Paper I);

2. to analyze the correlation of human blood isoAsp levels with dementia and AD progression (Paper II);

3. to further investigate the effects of isoAsp accumulation in the brain tissue of PIMT knockout transgenic mice (Paper III);

4. to search for blood prognostic biomarkers for AD among polypeptide abundances (Paper IV);

5. to probe the presence of early molecular signatures of AD in the presymptomatic AD mouse model (Paper V).

3 METHODOLOGY

3.1 Biomarker discovery by mass spectrometry

Mass spectrometry-based proteomics has been the driving force behind protein biomarker discoveries for the past decades. A modern mass spectrometer can routinely reach mass accuracy of 5 ppm at femtomole sensitivity, dynamic range of 3-4 orders of magnitude (up to six orders of magnitude in targeted analyses)[54] and identify thousands of proteins in a couple of hours.[55] However, it remains challenging to develop protein biomarkers in human blood, because of the large dynamic range (ten or eleven orders of magnitude), high complexity (more than 9,000 proteins) and wide interpersonal viabilities of blood proteome.[56] As a result, it requires extensive fractionation to cover the majority of the blood proteome, or using enrichment or depletion methods to reduce the dynamic range and focusing only on a subset of the whole blood proteome.[56]

The well-established protein biomarker discovery strategy is the "shotgun" proteomics, which profiles the proteome by analyzing the enzymatic digests of complex protein mixtures.[57] The digests composed of polypeptides are then separated by the liquid chromatography (LC) system coupled with a mass spectrometer. Mass spectrometry (MS) records the mass over charge ratio (m/z) of the polypeptide ions and their abundance. Every molecule with an appropriate m/z signature (e.g. Trypsin-digested peptides are typically doubly or multiply charged.) is selected for fragmentation by tandem mass spectrometry (MS/MS). The fragments contain sequence information, which is used for peptide/protein identification by database searching using search engine like Mascot, SEQUEST, or Andromeda. Both isotopic labeling (e.g. iTRAQ,[58] TMT,[59] and SILAC[60]) and label-free (e.g. spectral counting[61] and ion current integration[62]) quantification methods are used intensively in proteome quantification.[63] However, for biomarker discovery and animal model studies, isotopic labeling can become prohibitively expensive and impractical,[64] thus label-free protein quantification method was used throughout this thesis.[62] The quantified proteome data can then be subsequently submitted to various statistical software and bioinformatics databases and tools (e.g. REACTOME, KEGG, and DAVID) for further biological interpretation.

AD biomarker studies have mostly focused on proteins at low concentrations, e.g. cytokines, which cannot be easily accessed by MS-based methods. Thus the accuracy of the measured protein abundance suffers from low signal levels, which

reflects in the large (>>10%) coefficient of variability (CV).[65] In contrast, abundant blood proteins can be measured by label-free analysis with CVs as low as 1%, by which method, a panel of 20 abundant blood proteins can differentiate males from females from a single LC/MS analysis with ca. 90% accuracy.[62] Thus if AD progression perturbs the abundance of the top 100-200 abundant blood proteins ("top proteome"), one can hypothesize that a standard proteomic experiment that requires minimum sample preparation may suffice for AD prognosis with acceptable accuracy. Indeed, the third most abundant blood protein, α-2-macrogloblin (A2M), is viewed as one of the AD biomarker candidates.[66, 67] Therefore we analyzed the "top proteome" in search for biomarkers throughout this thesis.

3.2 Isoaspartate detection (Paper I)

Asp and isoAsp are structural isomers with identical molecular composition and similar physiochemical properties, and therefore it is quite challenging to distinguish the two by LC-MS. Several isoAsp detection methods have been developed, including Edman sequencing,[68] the ISOQUANT Isoaspartate Detection Kit (Promega) utilizing the enzyme activity of PIMT,[69] isoAsp specific antibodies,[70] endoproteinase Asp-N based method,[71, 72] affinity enrichment method,[73] isotopic ^{18}O labeling method,[74] and high performance liquid chromatography (HPLC) method.[75] MS/MS has also been explored for isoAsp identification. Collisional activated dissociation (CAD) was found to generate lower b/y ion ratio, less abundant b-H_2O, y-46 as well as Asp immonium fragment ions at the isoAsp site, but these findings were found to be sequence-dependent.[76, 77] Synthetic peptides have been investigated in the negative ion mode in electrospray MS as well as in the positive ion mode with higher-energy C-trap dissociation (HCD), but no specific signature for isoAsp has been found.[78, 79] In electron capture dissociation and electron transfer dissociation (ETD/ECD), the presence of isoAsp produces signature fragments of c•+58 and z'-57 ions (Figure 2).[80]

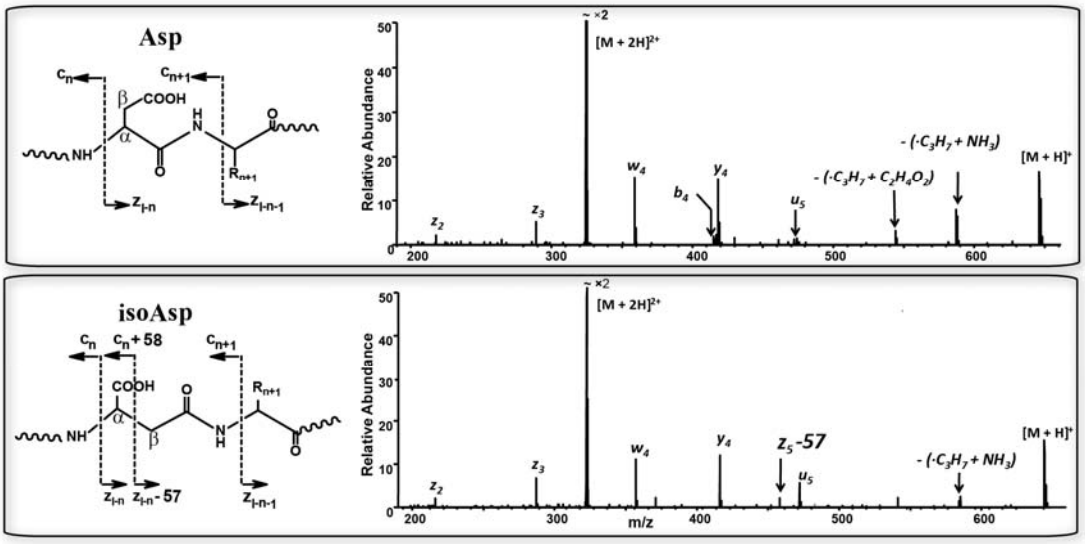

Figure 2. Left: ETD fragmentation pattern of a polypeptide containing Asp or isoAsp. The z'-57 and/or c•+58 fragments indicate the presence of isoAsp. **Right:** ECD MS/MS spectra of peptide LDLAGR 2+ molecular ions.

The main challenge for applying this method to analyze the biological complex mixtures is the fact that the peptides containing isoAsp are of low abundance. Therefore the signature fragments that are of even lower intensity can be drowned in noise. Sometimes the signature fragments overlap with isotopic distributions of other ions, which further complicate the detection of isoAsp. High resolution MS/MS can greatly help alleviate these problems.

In order to develop a suitable platform for proteome-wide isoAsp identification, we analyzed 32 human proteome samples by HPLC coupled with a 7 Tesla linear trap quadrupole (LTQ) Fourier transform mass spectrometer (Thermo Fisher Scientific). Each of the trypsin digested peptides that gave ions with $z \geq 2$ was fragmented by CAD as well as ECD. To test the specificity of isoAsp detection using the accurate mass of the signature fragments, high resolution was employed not only for peptide masses, but also for the detection of MS/MS fragments. From these samples, 466 isoAsp peptide candidates were identified based on the accurate mass of the signature fragments alone. However, many of the signature fragments from these isoAsp peptide candidates were found coming from spurious noise, overlapping with other fragment ions, or co-isolated ions in MS/MS spectra. Therefore, additional criteria were required to validate these candidates. These criteria include: complex chromatographic peak shape, presence of adjacent c and z fragments, high mass accuracy of 5 ppm, absence of specific Asp loss from the charge-reduced species, presence of the isotopic peaks of the signature

fragments, less abundant CO_2 loss from c, z fragments, presence of complementary specific fragments, and the same signature fragments found in multiple spectra.

Upon filtering with the above criteria, 219 isoAsp peptides have been confirmed by at least one criterion. Among these isoAsp peptides, 11 cases (five resulting from deamidation and six from isomerization) have been found as exceptionally convincing. Among the identified proteins, heat shock cognate, pyruvate kinase and actin have been identified earlier as substrates for PIMT.[81]

4 RESULTS AND DISCUSSION

4.1 Blood isoAsp accumulation in dementia patients (Paper II)

As mentioned, increased levels of isoAsp have been found in AD brain compared with healthy subjects,[52] and isoAsp has been detected in Aβ peptides from amyloid plagues.[50, 51] However, the role of other proteins bearing isoAsp damages in AD has not been explored. As increased levels of isoAsp have been found in blood samples of uremia patients who share some common pathology with AD patients,[33] we hypothesized that AD patients may also have higher level of isoAsp in their blood samples. Moreover, as females have higher prevalence for AD,[82] higher level of isoAsp in female blood should be expected compared to male blood.

To improve the detection sensitivity, we modified our previous proteome-wide isoAsp detection method (Paper I) by acquiring the ETD MS/MS spectra in the Velos linear ion trap. This resulted in fragmentation of many more peptides, but the benefits of high resolution detection of MS/MS fragments were lost. Therefore, two additional criteria were implemented to filter out erroneous isoAsp identifications. The criteria were: Mascot score >25, and the absence of mass overlap of the isoAsp signature fragments with any c or z type fragments. Moreover, to eliminate the artifact brought up by *in vitro* Asn deamidation, we focused on isoAsp originating from Asp isomerization only. Because the rate of Asp isomerization is much slower compared with Asn deamidation,[83] the majority of the detected isoAsp residues should originate *in vivo*.

218 blood plasma samples were pooled into eight groups based on sex (male, female) and disease progression (healthy subjects, S-MCI, P-MCI, and AD). These pooled samples were digested with trypsin and analyzed by MS/MS with HCD and ETD. The overall abundance of isoAsp in each sample was quantified by spectral counting method. The number of isoAsp-containing peptide MS/MS spectra was normalized by the total number of MS/MS queries in each LC/MS run. From the pooled samples, we found that MCI and AD patients have significantly ($p<0.05$) higher number of isoAsp counts (>10%) compared with healthy subjects, so do females (>10%) compared with males ($p<0.01$).

To validate the findings in the pooled samples, twelve individual, age-matched samples from each group were selected and analyzed in the same way as the pooled samples. The results for healthy subjects and dementia patients both from the pooled

and individual samples are shown in Figure 3A. The p-value for the isoAsp count differences between the dementia patients and healthy subjects was found by Fisher formula to be 0.01.[84] Similar results were obtained from females compared with males (Figure 3B).

Therefore, our hypothesis of both AD and sex effects on the isoAsp level in blood has been verified. The isoAsp-AD link is consistent with the previous findings for AD brains,[52] although the exact mechanism connecting these two findings remains unclear. The increased level of isoAsp in dementia and female blood samples can be hypothetically linked to the lower ratio of AdoMet to S-adenosyl-L-homocysteine in blood,[85-87] which potentially indicates lower repair efficiency by PIMT, assuming that its enzymatic activity exists in peripheral blood.

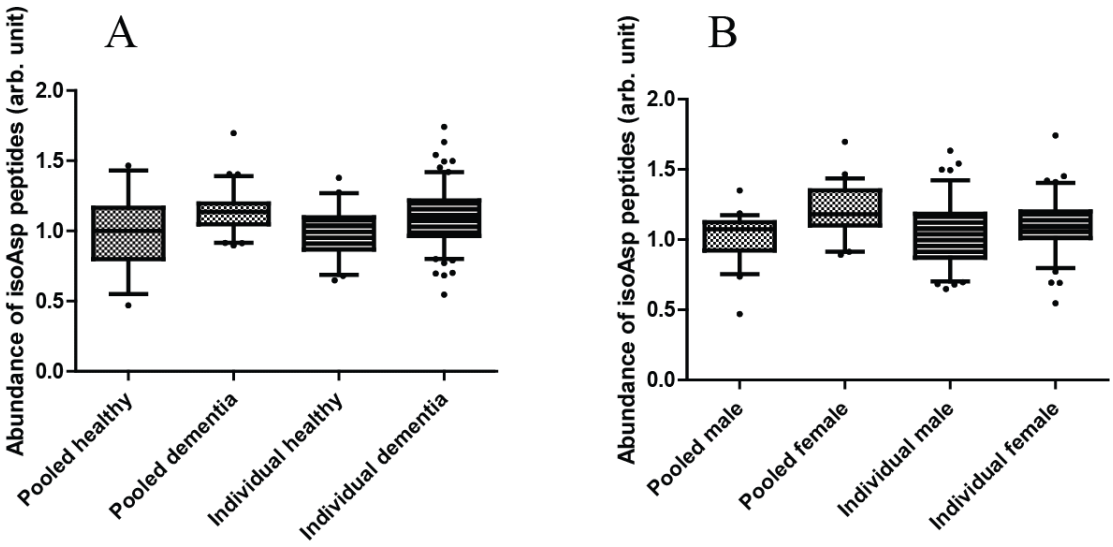

Figure 3. Box plot for the isoAsp abundance (counts) detected in human blood plasma: **A:** healthy subjects versus dementia patients; **B:** female subjects versus male subjects. Adapted from [29], with permission from IOS Press.

Although the excess of isoAsp counts in dementia patients is relatively low (around 15% on average), MS/MS spectral counts are logarithmically linked with peptide abundances,[88] and therefore the isoAsp level differences are likely to be higher than 15%. Furthermore, a single isoAsp modification in a protein can significantly influence the aggregation rate of Aβ peptides interacting with this protein.[50, 51] Thus isoAsp-containing protein species might play the "seeding" role in Aβ aggregation/amyloidogenesis in AD. And once the pathological processes are triggered, the blood isoAsp level becomes irrelevant (as long as it stays above the threshold),

which is consistent with our findings that the level of isoAsp does not scale with the severity of the disease.

4.2 Effects of brain isoAsp accumulation (Paper III)

To further explore the molecular effects of isoAsp accumulation, especially in the brain, we performed brain proteomic studies on a PIMT-KO transgenic mouse model. The deletion of the gene encoding PIMT in mice (*Pcmt1*) leads to significant isoAsp accumulation in the brain: the abundance of brain isoAsp in 30 to 40-day old PIMT-KO mice reaches six times higher levels compared to wild type (WT) mice, as measured by the ISOQUANT assay.[39] Abnormalities of microtubule organization in the pyramidal neurons, together with proportional brain enlargement (around 15% increase) in cerebral cortex, hippocampus, striatum, thalamus, and cerebellum, have been observed in these PIMT-KO mice, while there was no significant change in the number of neurons or astrocytes.[40] Finally these mice die prematurely of epileptic seizures.[39]

Eight female mouse brain tissues (four *Pcmt*1$^{-/-}$ and four *Pcmt*1$^{+/+}$) were analyzed in this study. We selected the in-solution filter-aided sample preparation (FASP) protocol that was not interfered by the large brain lipid contents.[89] To corroborate the findings provided by FASP, we performed an additional proteomics analysis with gel electrophoresis. Although mass spectrometry was used for protein identification and quantification in both analyses, such a validation approach was still satisfactory due to the complementary nature of peptide sets detected with FASP compared with in-gel digestion.[90] Moreover, this approach does not require significant changes of protein abundance (>50%) as required by the immunological validation methods.

The same MS/MS settings as in Paper II were applied here for isoAsp detection. While in this study, higher levels of isoAsp were expected in the PIMT-KO mouse brain, which reduced the concern over *in vitro* deamidation. Therefore, isoAsp originated from both deamidation and isomerization was investigated. In total, 67 unique isoAsp sites were identified. Four of the 12 isoAsp-containing proteins found by Vigneswara et al.,[41] and nine of the 19 isoAsp-containing proteins found by Zhu et al.,[81] were confirmed in our results. Moreover, we identified the specific sites of damage (isoAsp) in each of these proteins.

For isoAsp peptide quantification, reliable base-line chromatographic separation between the native and isoAsp peptides was required, which cannot always be achieved. Since the main goal was to assess the general isoAsp level in PIMT-KO and WT mouse brains, we selected two peptides as representatives with well-separated chromatographic peaks and reliable MS/MS identification of both native and isoAsp forms. The relative abundances of the variants were quantified by their extracted ion chromatogram. These two peptides are: ^{92}VFDKDG**N**GYISAAEL^{107}R from calmodulin and ^{65}LVI**N**GKPITIFQE^{78}R from glyceraldehyde-3-phosphate dehydrogenase (GAPDH), both of which were deamidation products. Their isoAsp occupancies (isoAsp/native peptide ratios) are shown in Figure 4. The relatively large amount of isoAsp in the WT mouse proteins suggests that these Asn residues may be particularly labile and thus could have deamidated during sample preparation.

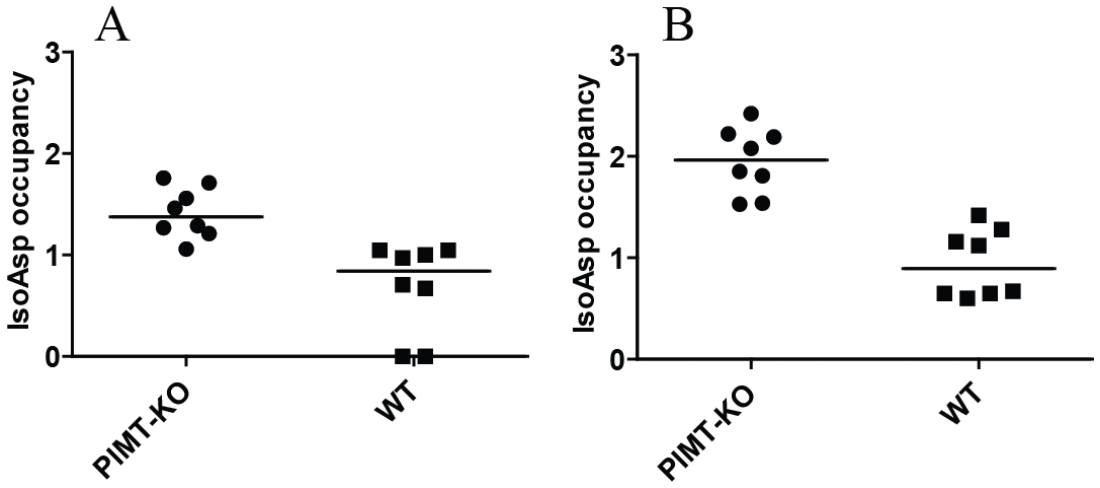

Figure 4. IsoAsp occupancy: **A:** peptide VFDKDG**N**GYISAAELR from calmodulin; **B:** peptide LVI**N**GKPITIFQER from GAPDH. Lines indicate the median value in each dataset. Reprinted with permission from [91]. Copyright (2013) American Chemical Society.

The peptide ^{92}VFDKDG**N**GYISAAEL^{107}R containing isoAsp-98 is located in the calcium binding loop of the calmodulin EF hand. Calmodulin contains four EF hand domains, all of which can bind calcium. Interestingly, the domain with Asn-98 has the highest calcium binding affinity among all domains.[92] The transformation of Asn-98 to isoAsp-98 in calmodulin results in loss of 90% protein activity.[93]

The domain which contains peptide ^{65}LVI**N**GKPITIFQE^{78}R bearing isoAsp-68 has no known catalytic activity of GAPDH, but it can bind tryptophanyl-tRNA synthetase,[94] and contribute to membrane fusion.[95] *In vitro* experiments have identified the N-terminal peptide (^{2}VKVGVNGFG^{11}R) as the main deamidation site

of GAPDH.[96] To our knowledge, this is the first time that GAPDH has been identified as a potential *in vivo* substrate for PIMT. The biological effects of deamidation of Asn-68 in GAPDH are still unclear. The chromatographic profiles together with the ETD MS/MS spectra for the native and isoAsp peptide ^{65}LVI**N**GKPITIFQE^{78}R are given in Figure 5.

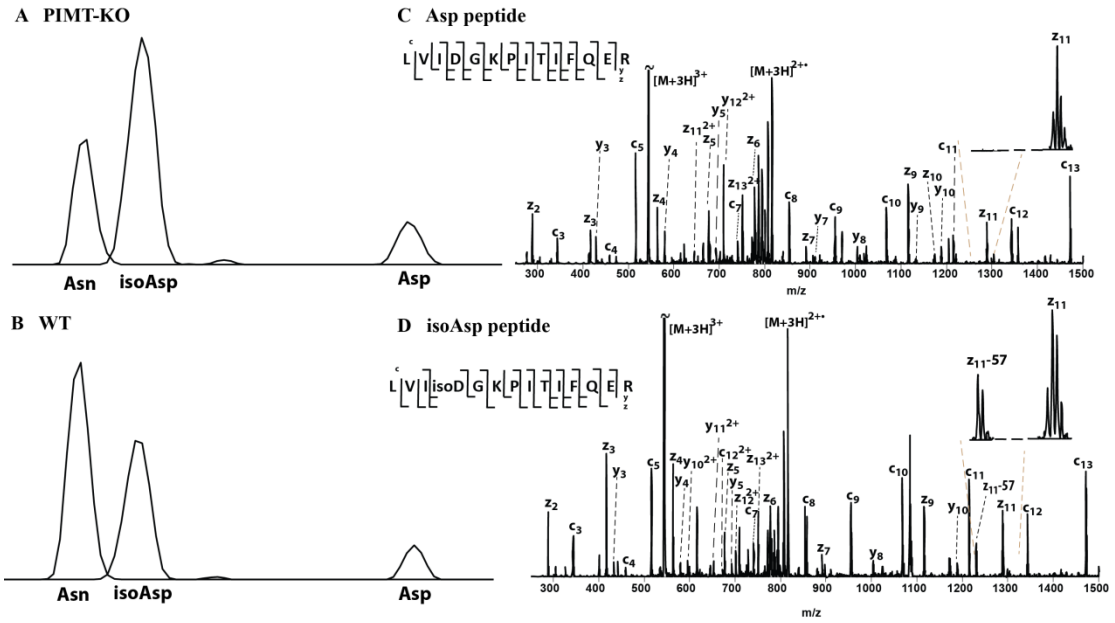

Figure 5. Left: Extracted ion chromatographic profiles of 3+ ions of peptide ^{65}LVI**N**GKPITIFQE^{78}R (Asn) from GAPDH and its deamidated forms ^{65}LVI**D**GKPITIFQE^{78}R (isoAsp) and ^{65}LVI**D**GKPITIFQE^{78}R (Asp) in the samples of **A:** PIMT-KO mice; **B:** WT mice. **Right**: ETD MS/MS spectrum of 3+ ions of **C:** the ^{65}LVI**D**GKPITIFQE^{78}R (Asp) peptide; **D:** the ^{65}LVI**isoD**GKPITIFQE^{78}R (isoAsp) peptide. Insert shows the absence or presence of (z–57 Da) ions. Reprinted with permission from [91]. Copyright (2013) American Chemical Society.

At the proteome level, among the most reliable 151 significantly regulated proteins, 83 were found to be up-regulated in PIMT-KO mice, and 68 down-regulated. Using all identified proteins as the background dataset and $p < 0.001$ as a significance threshold, Gene Ontology analysis yielded cellular amino acid biosynthesis as the only significantly enriched process. Moreover, the abundances of enzymes involved in the glutamate-glutamine cycle were altered towards the accumulation of glutamate,[39] which is consistent with the previous observations of 30% more glutamate in synaptosomes of PIMT-KO mice.[39] Excessive glutamate excitatory signals have been associated with epilepsy,[97] thus the dysregulated glutamate-glutamine cycle may

account for the epileptic seizures and, subsequently, of untimely death of PIMT-KO mice.

From our study, we confirmed that besides protein repair, PIMT has other regulatory effects, like in amino acid synthesis and glutamate-glutamine cycle. For the purpose of understanding the specific effects of isoAsp accumulation in the brain tissue, it will be of importance to carry out further functional studies of the proteins bearing significantly higher level of isoAsp in PIMT-KO mice.

4.3 AD blood prognostic polypeptide biomarkers (Paper IV)

In search for blood biomarkers of AD, we focused on the abundance changes of blood polypeptide for predicting AD progression in MCI patients. Blood plasma samples from 119 MCI patients (76 with S-MCI and 43 with P-MCI) were analyzed in two replicates (independent digestions) by the LC-MS/MS method mentioned in Paper II. For technical reasons, these samples were randomly assigned to be analyzed by two different LC systems in four series of experiments with at least two weeks break between each other. These experimental settings complicated the data analysis process, yet the obtained data were more representative of the real-life clinical settings. Relative abundances of the polypeptides were quantified by the label-free approach.[62] AD progression biomarkers were selected by multivariate analysis using SIMCA software (Version 13.0.0.0, Umetrics AB, Sweden) followed by cross-validation of the predictive model.

In total, 125 proteins were successfully quantified in all four groups of analyses. Orthogonal partial least squares discriminant analysis (OPLS-DA) model built for the discrimination of S-MCI and P-MCI groups showed predictive accuracy of 79%, with 60 proteins as the significant discrimination factors. In the negative control, where P-MCI/S-MCI identifier was randomly assigned, no valid OPLS-DA model can be built (Q^2 =-0.08).

Sex differences in human blood proteome are well known.[29, 62, 98, 99] For instance, the level of A2M in the blood is typically 15%-20% higher in adult females than in adult males.[100] According to the literature, A2M is an AD biomarker candidate.[66, 67] The overexpression of A2M in AD brains was found in 1991 based on immunohistological method.[101] Later, the 5′ splice-site deletion in exon 18 of A2M was genetically linked to AD with the same degree of certainty as the APOE-ε4 allele.[102] A 20% increase of A2M abundance in the blood has been found in AD patients compared with healthy subjects.[66] In our analysis, A2M level in the blood increased in P-MCI

females, but decreased in P-MCI males (Figure 6A). This observation is even stronger in the pooled data, where the same individual samples were pooled according to their sexes and disease progression.[29] In the pooled samples, A2M level in P-MCI females is higher than in S-MCI females by 13%, and in males the corresponding level is lower by 12% (Figure 6B).[62] This sex-specific correlation of A2M excluded it from the significant discrimination factors by the OPLS-DA model. But when an OPLS-DA model was built specifically for female patients, A2M was suggested as one of the strongest factors. Therefore, sex factor has to be assessed and evaluated for all the biomarker studies.

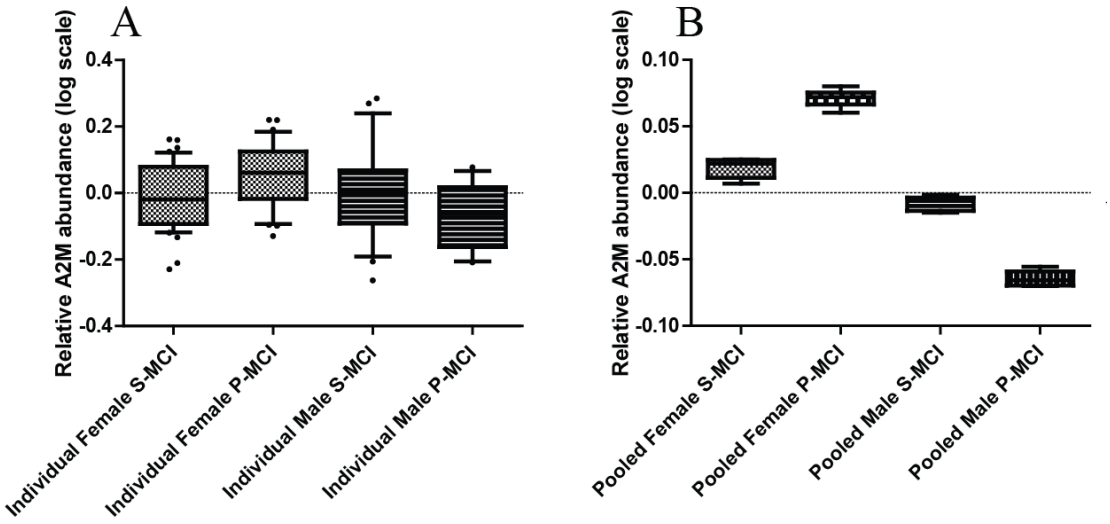

Figure 6. Box plot for the A2M abundance quantified from human blood plasma: **A:** abundances from individual samples; **B:** abundances from pooled samples.

Among the 60 significant discrimination factors, complement cascade was enriched from the P-MCI negatively-correlating proteins. The complement activation has been suggested as the mechanism for amyloid clearance and possibly served as biomarkers.[103] The involvement of complements here may reflect the inflammation aspect of the AD pathology and the response to it from the human immune system. On the other hand, hemostasis process was found enriched in the P-MCI positively-correlating proteins. This feature strongly supports the recently uncovered role of the hemostatic system and the clotting process in AD.[104]

As mentioned before, the current standard for discriminating P-MCI versus S-MCI by blood proteins exhibits the specificity and sensitivity around 80%.[24] Here, based on a panel of abundant blood proteins, we can achieve an accuracy of 79% in predicting AD onset (P-MCI). Among all the putative biomarkers proteins, ceruloplasmin,[105] complement factor I,[104] fibrinogen,[104] and plasma protease C1

inhibitor,[106] have been reported as increased in AD blood samples compared with healthy subjects.

This study is the first step to build a flexible and robust AD progression predictive tool that is based on blood biomarkers. All reliably detected and quantified proteins were used in building the AD model, which leaves space for future optimization and validation. For clinical application of these AD progression prediction models, further extensive validation is required.

4.4 Proteome alterations of presymptomatic AD mice (Paper V)

In the above study, the changes of the blood protein abundances were rather small, but still sufficient to predict AD progression at the stage of MCI. How early can the imminent AD be predicted by protein biomarkers then? To address this question, we performed the brain proteome studies of young TBA42 transgenic mice. As mentioned, the TBA42 mice expressing the "seeding" forms of Aβ, $A\beta_{pE3\text{-}42,}$ in the brain,[22] is a well-established model to investigate the early molecular events in AD. We chose to investigate the early proteome changes in the brain of four-month old presymptomatic TBA42 female mice. The proteomic experimental settings were similar as in Paper III.

As expected, the whole-brain proteome changes in the presymptomatic AD mice were small compared with the age-matched controls. Yet the main component in principal component analysis (PCA) of protein abundances separated the proteomes of WT and TBA42 mice rather well. This result confirms that the early signature of pathology is already present in brain of TBA42 mouse at four months of age, before the onset of detectable behavioral symptoms. Detailed studies found three proteins significantly upregulated in TBA42 mice:

- Cadm3 47 kDa protein, also known as nectin-like molecule 1 (Necl 1), is specifically expressed in the neurons. It is a Ca^{2+} independent immunoglobulin-like cell-cell adhesion molecule.[107] The connection between Necl 1 and AD has not been explored yet, and its importance for TBA42 neuropathology remains unclear.
- Homer1 protein belongs to Homer protein family. Its homologous proteins Homer2 and Homer3 interact with amyloid precursor protein and inhibit Aβ production, whereas Homer1 seems not.[108] The up-regulation of Homer1 might be mechanistically related to the cognitive deficits that TBA42 mice develop at a later time point.[109]

- Inositol-trisphosphate 3-kinase A ($InsP_3KA$) converts inositol-1,4,5-trisphosphate ($InsP_3$) to inositol-1,3,4,5-tetrakisphosphate in hippocampal, cortical and cerebellar neurons. Thus it is an important regulator of $InsP_3$ induced calcium signaling pathway.[110] Calcium signaling is intensively involved in neuron physiology, and enhanced intracellular calcium levels have an essential role in AD.[111] The increased $InsP_3KA$ levels in young TBA42 mice may function as to decrease the $InsP_3$ level, thus restoring the calcium balance.

To put the proteome results in the context of signaling pathways, quantitative pathway analysis was performed using pathway search engine with TRANSPATH database.[112] Briefly, protein IDs are converted into gene IDs, and mapped on known signaling pathways from the TRANSPATH database.[113] Then an upstream search along the pathway networks is performed, the regulatory molecules on the pathway intersections (so called key nodes) are scored according to their connectivity to the input proteins and abundances.[113]

Among all the significantly upregulated key nodes in TBA42 mice, three nodes were localized in mTOR/p70S6K anti-apoptotic signaling pathway. The mammalian target of rapamycin (mTOR) plays a pivotal role in multiple fundamental biological processes. A variety of reports suggested that the mTOR pathway modulates Aβ-related synaptic dysfunction in AD.[114] The relevance of mTOR/p70S6K pathway in AD has also been confirmed by studies with Tg2576 AD transgenic mice,[115] primary neuron culture from R1.40 mice,[116] and in the brains of 3xTg-AD mice.[117] Our results of increased mTOR signaling in young TBA42 mice is corroborated by these previous findings and further suggest that this pathway may be involved in presymptomatic neurodegeneration.

Our data confirm that AD-typical molecular pathways can be detected by whole-brain shotgun proteomics in young presymptomatic mice long before the onset of behavioral changes.

5 CONCLUSIONS

From the above studies, we have demonstrated:

1) for the purpose of proteome-wide detection of isoAsp in biological complex mixtures, the presence of the isoAsp signature fragments in ECD/ETD spectra in combination with additional criteria (either form MS or LC) are efficient for isoAsp identification;
2) higher levels of isoAsp have been detected in blood plasma samples of dementia patients compared with healthy subjects, and females compared with males;
3) besides isoAsp repair, PIMT has other regulatory effects such as a role in amino acid synthesis and glutamate-glutamine cycle in the brain;
4) based on the top proteome of around 120 blood protein abundances, the prediction accuracy for the progression of MCI patients to AD can reach 79 %;
5) AD-related molecular pathways can be detected in young presymptomatic transgenic AD mice long before the onset of behavioral changes.

6 FUTURE PERSPECTIVES

The updated AD Diagnosis Guidelines from the National Institute on Aging have suggested implementing brain imaging and CSF biomarkers in 2011.[118] These biomarkers have already reached (e.g. Florbetapir) or approached clinical application. However, due to their relatively high cost and invasive sampling procedures, blood biomarkers are not only highly desirable for prognosis, diagnosis, and disease onset/intervention monitoring, but also for population screening or multistage screening.[119] Therefore, this thesis was dedicated to search for AD blood biomarker candidates.

All the biomarker studies carried out in this thesis are in the discovery phase, which was based on unbiased detection of protein expression and posttranslational modification differences in blood samples of the patients with different degrees of dementia. Before clinical use, these results will require further validation through independent analytical systems: ideally, using other than MS method, and preferably on thousands of samples. Thus before extensive validation is finished, all these findings should be regarded as "biomarker candidates".[120]

Since blood reflects the metabolism alterations from all tissues, the detected blood proteome disturbances may result from pathological processes indirectly linked to AD brain pathology. Therefore, the biomarker candidates that are either brain-specific or identified both in CSF and blood could be more promising as the final biomarkers. Since the definite AD diagnosis still relies on postmortem brain examination, all current biomarker studies based on clinical diagnosis are limited by the accuracy of that diagnosis. Furthermore, there are many sources of potential analytical variations between different laboratories and clinics resulting from the differences in sample collection, transportation, storage and handling before the analysis. Therefore, standardization is required for a multi-cohort validation study. Validation of predictive biomarkers (P-MCI) is especially challenging as it requires relatively long follow-ups to corroborate the current diagnosis.[119] As for the preclinical AD biomarkers development, well-designed longitudinal studies are needed. One such study has recently been launched by our group in collaboration with other KI groups and supported by AstraZeneca. The results of the current thesis will serve a basis for analytical proteomics approaches in that study.

Besides the development of AD blood protein biomarkers, we have also identified elevated levels of protein damages caused by isoAsp accumulation in MCI and AD patients. We hypothesized that isoAsp-containing proteins may play the "seeding" role in amyloidogenesis in AD. Although elevated levels of isoAsp-containing proteins have been found in AD brain, their identities remain unknown. Therefore further investigations into the AD brain isoaspartome will facilitate the understanding of the protein ageing theory of Alzheimer's disease.

7 ACKNOWLEDGEMENTS

I would like to take the opportunity to express my appreciation to all those who have contributed to this thesis and been supportive through my work.

In particular, I am indebted to my principal supervisor Prof. **Roman A. Zubarev** for his encouragement, inspiration, guidance and support through the years. I am grateful for all the challenges, freedom, as well as the opportunities given by him.

I would like to express my appreciation to Prof. **Neus Visa** and Prof. **Agneta Nordborg**, my co-supervisors, for always being supportive. I am grateful to my mentor Prof. emeritus **Jan Sjövall**, who always cares about my project progresses.

I would also like to thank Prof. **Steven Clark**, Prof. **Thomas Bayer**, and Prof. **Philippe Grappin**, who initiated collaborations with us after my first publication, together with Dr. **Javier González**, Dr. **Mikhail Gorshkov**, Dr. **Jonathan Lowenson**, you have brought broader prospective to my Ph.D. studies!

I want to give gratitude to the following people in the lab: to **Yaroslav** for making my life much easier with computer programming; to **Consuelo** for helping me with wet lab experiments; to **Jimmy** for being always available and helpful with project discussions; to **David G.** for sparing me the knowledge of instrumentation; to **David B.** for teaching me chemistry principles; to **Susanna** for sharing knowledge of glycosylation. My sincere thanks also go to PK/KI: **Doro**, who is always efficient with instrument troubleshooting and giving critical suggestions; **Marie S.** and **Carina**, who have been quite helpful for the Alzheimer's studies as well as everyday lab life; **Gunvor** and **Marie D.** for instrumentation issues. Stor kram!

I would like to thank my ***Spanish Mafia***: **Marta**, **Consuelo**, **David B.**, **Juan**, **Toni**, **Ernesto**, and **Daniel**, for the fantastic trips in south Spain, the amazing *marinera* and *tinto de verano* recipes, friendship at the foosball table, and their warm companionship and encouragement.

I appreciate my colleagues in the Zubarev group: **Eva Fung**, **Konstantin A**, **Corina**,

Therése, **Pierre**, **Bo Zhang**, **Mohammad**, **Konstantin C.**, **Nataliya**, **Harleen**, **Alexey**, **Oleksii**, **Chao Sun**, **Harsha**, **Atim**, **Anwer**, **Liban**, and **Xueshu**. You guys have made the lab a really pleasant and supportive place to work in! Many thanks go to **Victoria**, thank you for being always friendly and helpful with administrative issues!

I also want to acknowledge *the Wheelock boys*: **Anas**, **Stuart** and **Marcus**, for the enjoyable time we spent during lunch break and sporadic knowledge of metabolomics.

I appreciate my friends at MBB: **Jianqiang**, **Weiping**, **Xun Wang**, **Meifong**, **Jia Sun**, **Xu Zhang**, **Qing Cheng**, **Ying Sun**, **Jun Lu**, **Linus**, and **Essam**. Without you guys, my studying in the department would have been much less fun.

Special thanks go to **Huilin**, **Terry**, **Joanna**, and **Cuihong**: we appreciate the beauty of *mass spectrometry* as well as *hiking, skiing, snorkeling, and other travelling adventures* together. I am lucky to have you guys.

I have to express my appreciation to: **Yang Chen**, **Yumeng** and **Yashas**, for our long-lasting friendship; **Suman**, for being always supportive; **Mengmeng** and **Shiying**, who are quite cheerful people and took care of my kitty while I was not in town.

I would like to express my love for my darling kitty: ***MiuMiu***, with whom I had tons of fun. She has taught me the feelings of being responsible and caring.

最后我要十分感谢我的父母，是你们带我来到这个世界，给了我多年来的自由和最大程度的支持。

Finally, I would like to thank Prof. **Roman Zubarev**, **Jimmy**, Prof. **Agneta Nordborg**, and Prof. **Thomas Bayer** for the suggestion and correction of this thesis.

These four years have been an exciting and unforgettable adventure of my life. I am so grateful to have you all along this path with me!

Hongqian, 2013@Stockholm

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